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Abstract

For many years, reports have been published describing the discovery and investigation of an additional vascular system in mammals. This primo vascular system (PVS) is distinct from the blood and lymph vascular system and consists of primo nodes (PNs) and primo vessels (PVs) as its main constituents. We investigated samples of the PVS from the intestine surface of rats and observed several instances of a red thread-like structure (RTLS) and a red oval or round structure (RORS) in PVs and PNs, respectively. We conclude that the RTLS and RORS are most likely due to erythrocytes, indicating the occurrence of extramedullary hematopoiesis inside the PVS of the intestine surface. To the best of our knowledge, this is the first report showing detailed microscopic images of an RTLS traversing four PNs and a single PV. Our report is intended to document our findings and also to motivate others to repeat and extend our study in order to investigate in detail the possible extramedullary hematopoiesis occurring inside the PVS.

Introduction

Do mammals have another vascular system distinct from the blood and lymph vascular system? For many years, researchers, mainly from Asia, claim to have discovered such a system. The initial report came from the North-Korean researcher Bong-Han Kim in the 1960s, and parts of discoveries described in this report were rediscovered decades later by the South-Korean research group of Kwang-Sup Soh [1]. There is currently growing international interest in this topic. Since 2010, the novel anatomical structure is termed "primo vascular system" (PVS) comprising "primo vessels" (PVs) and "primo nodes" (PNs), distributed throughout the entire body [1]. The PVS has been detected in several different types of animals as well as in different tissues and systems, e.g. at the surface of organs [2] [3] [4] [5] [6] [7] [8], the inner abdominal wall [9] [8] [10], inside and along blood vessels [11] [12] [13], inside lymphatic vessels [14] [15] [16] [13] [17] [18] [19] [20], in the endocardia of atrium and ventricles of the heart [11] [21], in adipose tissue [22], along the central canal [23] and around the perineurium of the spinal cord [24], in the cerebrospinal fluid of the brain ventricles [23], in the arachnoid mater of the brain [24], along the sciatic nerve [25], in the fascia along skin skeletal muscles in the hypodermal layer [26], as well as at the surface of the umbilical cord [27] and placenta [27] [28]. These reports found that the thickness of the PVs is about 20–150 µm with a length of up to several centimeters. There is a liquid flowing in PVs [7], and the PVs is comprised of subducts and sinuses (i.e., tubular structures inside the PVs and PNs) [3]. Several studies demonstrated that the PVS is different from blood vessels, lymphatic vessels or nerves with respect to immunochemistry, histology and genetics [25] [29] [9] [4] [19] [30] [31] [8], making a strong argument that is a unique anatomical structure/system. Concerning the function of the PVS, the research so far indicates roles in immune function [32], tissue regeneration [33] [34], erythropoiesis [35] [36], as a niche and possible transport route for stem cells or stem cell-like cells [37] [33] [34] [38], and as a transport route for microvesicles and exosomes [39] [40]. Also, a route for metastasis in the case of cancer has been reported [10].

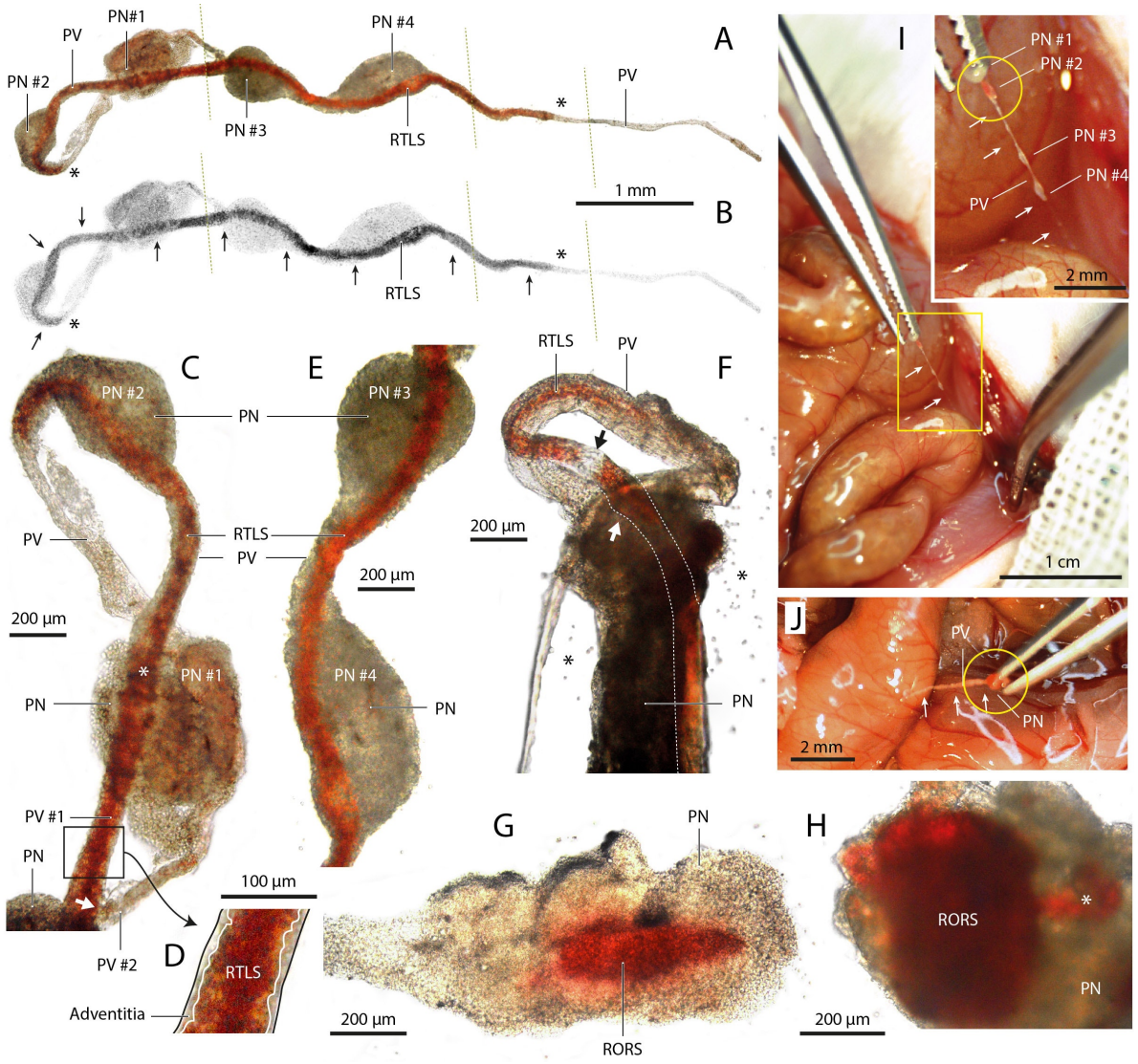
A summary of the current knowledge about the PVS can be found in a few review articles [41] [42] [43] [44] and a monograph [1].

Despite the many investigations of the PVS published by different research groups in

animals (e.g. rats, rabbits, cows, mice, dogs, pigs) and even samples of human tissue (umbilical cord and placenta [27]) with modern histological, immunochemistry and diverse microscopic techniques, the PVS is not well known by Western scientists, or if known, is generally viewed with skepticism. The lack of publications about the PVS in high-impact scientific journals as yet, the publication of the research results often in alternative medicine journals, as well the current lack of research on the PVS by Western scientists, seem to be the main reasons for it. In addition, the fact that the PVS is semi-transparent and can be easily misidentified as "connective tissue" (personal communication and experience) might explain why it is not normally detected during surgical examinations. There is a need for a novel investigation into the PVS by experimental work that revaluates the published claims and sheds new light on the anatomy and function of the PVS.

Objective

As part of a one-month research stay in South Korea, the first author had the chance to work at one of the most active and experienced research groups concerning the PVS, i.e., the group of P.-D. Ryu at Seoul National University (SNU) in South Korea. During this stay, the author learned to detect, extract, and analyze the PVS from abdominal organ surfaces of rats. One objective of the research was to perform a detailed analysis of PVS specimens with phase-contrast microscopy and digital image analysis. The results, with a focus on one particular discovery, are reported here.



a

Figure Legend

Figure 1. Microscopic images of PVS samples with red-colored structures (red thread-like structure, RTLS; red oval or round structure, RORS) from the intestine surface, and images from the extraction process.

(A) PVS specimen with four PNs connected by a single PV. The image was created by stitching together four single microscopic images (transitions between the images are marked with vertical lines). The red part of the PV is the RTLS. The whole extracted structure is about 1 cm long. The red color channel in the HUE color representation of the image was increased from 0 to 100 to increase the visibility of the RTLS. The start and endpoints of the RTLS are marked (*).

(B) Result of segmenting image **(A)** into the three color channels (R, G, B) and creating a new image by subtracting the red channel from the green one. With this, the visibility of the PEC is increased and the PEC is easily visible in the grayscale image as a dark threadlike structure.

(C, D, E) Magnified sections of the PVS specimen shown in **(A)**. In **(C)** the RTLS is going through PN #2. PN #2 is behind the PV indicated by an asterisk, i.e., it is not traversing this PN. The white arrow in **(C)** is indicating a section where two PVs seem to merge.

This is, however, not the case: PV #2 is behind PV #2 and the arrangement of the two PVs is only caused by the placement of the whole PVS sample on the glass slide. One part of **(C)** is magnified in **(F)**, showing the ultrastructure of the PV with an adventitia and the RTLS inside the inner part of the PV. In **(E)** the RTLS is traversing both PNs.

(F) PVS specimen with a PN and a PV with a RTLS inside. The possible route of the RTLS inside the PN is indicated with white dotted lines and a white arrow. The black arrow indicates a region of the PV where there is no RTLS visible, i.e., there is a discontinuity of the RTLS. Around the PN, several single small cells (having a diameter of about 10 µm) are visible (*). The red color channel in the HUE color representation of the image was increased from 0 to 100 to enhance the visibility of the PEC.

(G, H) PVS specimen consisting of a PN with a RORS located inside the PN. The red color channel in the HUE color representation of the image was increased from 0 to 30 **(G)** and 40 **(H)**, respectively, to enhance the visibility of the RORS.

(H, I) Photographic documentation of detected and removed PVS samples from the intestine surface of a rat.

The figure with a higher resolution is attached.

Results & Discussion

From the five rats investigated, several parts of the PVS were identified in the abdominal cavity (at the surface of the intestine, the liver, and the abdominal wall). In general, half-transparent, milky-white, spots (PNs) were first found on the surface of the tissue and then picked by a tweezer. When the PNs were picked and moved, a vessel (a PV) was recognized in all instances as they were attached to the PN. In each rat, multiple PVS samples were taken and subsequently investigated under the microscope. The diameter of the PVs was in the range of approx. 50–150 µm.

This manuscript focuses on reporting one specific finding: red-colored parts of the PVS samples that were seen either directly without magnification when extracting the PVS parts (see the red PN in Fig. 1I, J) or under the microscope (Fig. 1A-H). One sample obtained with this characteristic consisted of four PNs and a PV of a total length of about 1 cm (Fig. 1A-E). The investigation of this sample under the microscope showed a surprising feature: a red tubular-like structure passing through the PN and all four PNs (Fig. 1A-E). The same feature was seen in another sample comprising a PN and a PV (Fig. 1F), and two samples of PNs (Fig. 1G, H). Also, two different PNs extracted exhibit red parts at their center (Fig. 1G, H). Thus, two types of red structures were found: a *red thread-like structure* (RTLS) and a *red oval or round structure* (RORS). An RTLS was observed passing thought PNs and a PV (Fig. 1A-F), and several RORSs were found to be located at the center of PNs (Fig. 1G, H).

Figure 1I and J show two instances where PVS samples were taken from the intestine surface. In both instances, a red-colored PN was first observed on the surface of the abdomen and was then pulled-out with the tweezer, revealing a PV attached to it. The sample shown in figure 1H corresponds to the PN shown in figure 1J, while the sample depicted in figure 1I is not shown as a microscopic image.

Figure 1D shows a zoomed-in part of the PV, clearly showing that the RTLS is an inner structure in the PV, possibly surrounded by an adventitia.

Since hemoglobin is the only red-colored chromophore available in the tissue of rats, the RTLS inside PVs and the RORS in PNs are most likely caused by the presence of erythrocytes. According to our view, there are three principle reasons for this. (i) The erythrocytes could be due to contamination of the PVS samples with blood coming from the surgical procedure. (ii) The PVS serves as a transport route for erythrocytes coming from a connection between the PVS and vascular blood vessels. (iii) The erythrocytes are produced inside the PVS as a form of extramedullary hematopoiesis. Possibility one can be discarded since the surgical procedures were performed carefully so to not contaminate the PVS samples with blood, and the characteristics of the red-colored parts

are not in line with contamination: the RTLS is a continuous red line *inside* the PV, and the RORS is a part *inside* the PN. If contamination were the cause, erythrocytes would have also been on the outside of the extracted PVS samples. The second possibility cannot be discarded and might be true, in principle. Possibility three, however, seems to be the most likely one. The following reasons support this conclusion: (i) if extramedullary hematopoiesis takes place inside the PVS, there would be red parts inside (and not outside) the PVs and PNs, as observed. (ii) Previous studies showed that the PV contains a sinus and sub-PVs (s-PVs) that function as transport routes for a fluid (primo fluid, PF) as well as a large variety of macromolecules and cells, including stem cells [37] [33] [30]. Specific cells (diameter: 3–5 µm) inside the PV and PN tested positive for expression of stem cell markers CD133, Oct3, Oct4, Nanog, SSEA, Sox2 [37] [33] [34]. (iii) At least four publications reported already about the presence of erythrocytes in samples of the PVS. Choi et al. [45] microscopically observed cells with an appearance of erythrocytes in PN slices (see Fig. 1(B) in their paper). Han et al. [46] also observed cells in the shape of erythrocytes in PN slices under the microscope (see Fig. 1(D) in their paper). Our group at SNU [5] confirmed the presence of erythrocytes in PN slices by optical microscopy and fluorescence microscopy (hematoxylin and eosin (H&E) and hemacolor staining) (See Fig. 6 of their paper). In another paper by our group at SNU [35] we reported also the observation of RTLS and RORS parts of PVs and PNs, respectively (see Fig. 53.1(d) in their paper) and provided evidence for the presence of erythrocytes in PVS samples from the intestine surface by H&E and hemacolor staining as well as transmission electron microscopy. We also showed that the occasions of PVS samples found with RTLS and RORS parts were higher in rats with heart failure (associated with anemia) compared to controls. We concluded that extramedullary hematopoiesis inside the PNs and PVs erythropoiesis is happening. In addition, we detected reticulocytes in the PVS samples, adding further support to the notion of extramedullary hematopoiesis (and erythropoiesis, in particular) happening in the PVS. This conclusion is supported by the finding of Kim et al. [37] reporting the expression of the hematopoietic stem cell marker Thy 1 of cells inside the PVS.

The half-transparent, milky-white, spots (i.e., the PNs) are not Peyer's patches (PPs). Compared to PNs, PPs are much larger in size and do not have a vessel connected the the the primo vessel. In addition, unlike the PVS tissue, the PP is based on the submucosal layer of the intestine, and so one cannot isolate the PP with its intact gross morphology like the PVS tissue.

To the best of our knowledge, a continuous red line (RTLS) traversing PNs and seeming to be inside the PV has not yet been documented, and our report is the first one providing microscopic images of this specific morphological aspect of the PVS.

Conclusions

In multiple instances, we observed red-colored parts of the PVS (i.e., RTLS inside PVs and RORS inside PNs) by microscopic analysis of PVS specimens extracted from the intestine surface of rats. According to the indications discussed, we conclude that the RTLS and RORS are most probably due to erythrocytes present in PVs and PNs, indicating extramedullary hematopoiesis inside the PVS.

Limitations

This study has two main limitations. First, only a limited number of the PVS samples investigated showed the specific features of an RTLS inside PVs and a RORS inside PNs, meaning it was not possible to provide more examples of this histological feature. Secondly, we used only optical microscopy in our study. Additional investigation of the PVS specimens with immunohistochemistry would have been advantageous, and future studies should perform such analysis.

Alternative Explanations

Conjectures

Further studies should replicate and extend our findings with more PVS samples and by applying immunochemistry analysis to prove the existence of erythrocytes and hematopoietic stem cells in the PVS from the intestine surface as well as the PVS from other parts of the organism. It should be investigated if the possible extramedullary hematopoiesis happening in the PVS of the intestine surface is also occurring in other subtypes of the PVS, e.g., the PVS along/in blood vessels or lymphatic vessels. The ultrastructure of the PVS should also be studied in detail since previous studies already discovered subvessels of the PVS [3] [5], which may act as transporting routes for different components. Maybe one specific type subvessel of the PVS is especially linked to extramedullary hematopoiesis. The study should also be extended to find RTLS inside PVs and RORS inside PNs also in other mammals, including human tissue. If confirmed, the discovery of extramedullary hematopoiesis might have a significant impact in the field of hematology by indicating that the PVS is involved in hematological functioning of an organism. The exact role of the PVS for this aspect then needs to be investigated systematically. Since extramedullary hematopoiesis has already been linked to inflammation and infection [47] [48] [49] [50], and since the PVS is also shown to have an immune function, the extramedullary hematopoiesis of the PVS appears to be an adaptive mechanism to inflammation and infection.

Additional Information

Methods

Male Sprague-Dawley rats (Orient Bio, Gyeonggi-do, Korea; $n = 5$, age: 5–7 weeks) were used in this study. The animals were housed in a temperature-controlled room (20–26℃) under a 12 h light/dark cycle with food and water available ad libitum.

Rats were anesthetized by an intramuscular injection of an anesthetic cocktail (alfaxalone, 41.7 mg/kg, intraperitoneally; xylazine. 16 mg/kg, intraperitoneally). For each measurement, the anesthetized rats were placed under a stereomicroscope (OSM-1, Dongwon, Seoul, Korea) and the abdomen was opened. The surgical procedure was carefully conducted so as not to cut blood vessels and to stop minimal bleedings immediately to avoid the blood entering the abdominal cavity. With two tweezers, the surface of the intestine was then searched for semi-transparent threadlike structures (i.e. PVs) and semi-transparent spots (i.e. PNs). The search was conducted about 10 min after opening the abdominal cavity to let the fluid covering the organs evaporate to make detection easier. If PVs or PNs were found, they were removed gently with tweezers and were placed in a Petri dish with phosphate-buffered saline (PBS). Afterward, the samples were analyzed under a phase-contrast microscope (Olympus inverted microscope, IX70) while the PVS samples were analyzed in a wet state, i.e. immersed in a few drops of PBS to prevent the tissue from dehydration. Image processing and analysis was conducted with GIMP 2.10.0 and ImageJ 1.52a.

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Ethics Statement

The experimental protocols involving animals were approved by the Local Ethics Committee.

The animal experiments performed were in accordance with the guidelines of the Laboratory Animal Care Advisory Committee of Seoul National University and were approved by the Institute of Laboratory Animal Resource of Seoul National University (SNU-140926-2).

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